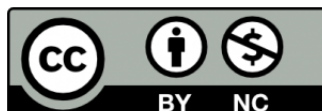




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# Salivary and serum cortisol levels, salivary alpha-amylase and unstimulated whole saliva flow rate in pregnant and non-pregnant

*Níveis de cortisol salivar e sérico, alfa-amilase e fluxo de saliva total não estimulada em gestantes e não gestantes*

## Original Article

### Keywords

Saliva  
Cortisol  
Alpha-amylase  
Pregnancy  
Menstrual cycle

### Palavras-chave

Saliva  
Cortisol  
Alfa-amilase  
Gestação  
Ciclo menstrual

### Abstract

**PURPOSE:** To compare salivary and serum cortisol levels, salivary alpha-amylase (sAA), and unstimulated whole saliva (UWS) flow rate in pregnant and non-pregnant women. **METHOD:** A longitudinal study was conducted at a health promotion center of a university hospital. Nine pregnant and 12 non-pregnant women participated in the study. Serum and UWS were collected and analyzed every trimester and twice a month during the menstrual cycle. The salivary and serum cortisol levels were determined by chemiluminescence assay and the sAA was processed in an automated biochemistry analyzer. **RESULTS:** Significant differences between the pregnant and non-pregnant groups were found in median [interquartile range] levels of serum cortisol (23.8 µL/dL [19.4–29.4] versus 12.3 [9.6–16.8],  $p<0.001$ ) and sAA (56.7 U/L [30.9–82.2] versus 31.8 [18.1–53.2],  $p<0.001$ ). Differences in salivary and serum cortisol (µL/dL) and sAA levels in the follicular versus luteal phase were observed ( $p<0.001$ ). Median UWS flow rates were similar in pregnant (0.26 [0.15–0.30] mL/min) and non-pregnant subjects (0.23 [0.20–0.32] mL/min). Significant correlations were found between salivary and serum cortisol ( $p=0.02$ ) and between salivary cortisol and sAA ( $p=0.01$ ). **CONCLUSIONS:** Serum cortisol and sAA levels are increased during pregnancy. During the luteal phase of the ovarian cycle, salivary cortisol levels increase, whereas serum cortisol and sAA levels decline.

### Resumo

**OBJETIVO:** Comparar os níveis de cortisol sérico e salivar, alfa-amilase salivar (sAA) e fluxo de saliva não estimulada (UWS) em gestantes e não gestantes. **MÉTODOS:** Trata-se de um estudo longitudinal realizado no centro de promoção da saúde de um hospital universitário. Nove gestantes e 12 não gestantes participaram do estudo. Foram coletados e analisados soro e UWS nos três trimestres gestacionais e duas vezes por mês durante o ciclo menstrual. A análise do cortisol salivar e sérico foi realizada com o uso de quimiluminescência e a atividade da sAA foi determinada por meio de analisador automático para bioquímica. **RESULTADOS:** Foi verificado que a mediana (intervalo interquartil) dos níveis de cortisol sérico no grupo de gestantes foi maior que 23,8 µL/dL (19,4–29,4) quando comparado ao grupo de não gestantes, que teve média de 12,3 (9,6–16,8;  $p<0,001$ ). Os níveis de sAA seguiram o mesmo padrão, com médias de 56,7 U/L (30,9–82,2) e 31,8 (18,1–53,2;  $p<0,001$ ), respectivamente. Foram observadas diferenças dos níveis de cortisol sérico e salivar (µL/dL) e de sAA entre a fase folicular versus a fase lútea ( $p<0,001$ ). As medianas dos fluxos salivares (UWS) foram semelhantes em gestantes (0,26 [0,15–0,30] mL/min) e não gestantes (0,23 [0,20–0,32] mL/min). Foram encontradas correlações significativas entre o cortisol salivar e o sérico ( $p=0,02$ ) e entre o cortisol salivar e a sAA ( $p=0,01$ ). **CONCLUSÕES:** Os níveis de cortisol sérico e sAA durante a gestação elevam-se. Na fase lútea do ciclo ovariano, os níveis de cortisol salivar aumentam ao passo que os níveis de cortisol sérico e sAA diminuem.

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Conflict of interests: none.

## Introduction

Laboratory analysis of saliva has become an important technique for the assessment of physiological and pathological conditions, mostly due to the origin, composition, and functions of saliva, as well as its interactions with other body systems and structures. Other favorable aspects of saliva testing include painless sampling, ease of storage, and low cost of analysis as compared with blood. These factors have driven extensive research into this testing modality<sup>1-3</sup>, including validation studies of quantitation of a variety of organic and inorganic compounds in saliva<sup>4</sup>.

Cortisol is a hormone secreted by the adrenal glands that can be detected in urine, serum, and saliva. Measurement of cortisol levels in saliva is gaining increasingly widespread acceptance as a diagnostic method because they correspond only to the unbound, bioactive fraction of cortisol, whereas most serum cortisol is bound to proteins such as corticosteroid-binding globulin (CBG)<sup>5,6</sup>. Salivary cortisol testing has been used to assess hypothalamic-pituitary-adrenal (HPA) axis function under various cognitive conditions and in the presence of stress and anxiety<sup>6,7</sup>. During pregnancy, baseline salivary cortisol concentrations exhibit a constant increase starting around gestational week 25; by term, levels are over twice as high as those detected in non-pregnant women<sup>8</sup>. Within one week after delivery, salivary cortisol levels returns to baseline<sup>8</sup>. The physiology of cortisol can be assessed under baseline conditions and in response to specific stressors<sup>9,10</sup>. Measurement of changes in baseline cortisol levels and in cortisol reactivity to stress during pregnancy is important, as high concentrations of cortisol affect fetal development<sup>7,10,11</sup> and may lead to low birth weight<sup>12</sup>.

The enzyme salivary alpha-amylase (sAA) is one of the key protein constituents of saliva and accounts for 10–20% of all proteins produced by the parotid gland<sup>13</sup>. Its function includes, but is not restricted to, initiation of digestion in the oral cavity. It also plays a major role in modulation of bacterial adhesion and growth on intraoral surfaces<sup>14</sup>. Recent studies have highlighted the utility of sAA as a marker of physical, psychological, or psychosocial stress induced by activation of the autonomic nervous system, which controls the salivary glands<sup>13,15-17</sup>. Furthermore, increased levels of sAA have been shown to reduce the likelihood of conception during the fertile window in women<sup>18</sup>.

Pregnancy-related changes in sAA secretion have rarely been described in the literature. Studies suggest that salivary flow and sAA levels remain unchanged during gestation<sup>19,20</sup>. However, a research has also shown that pregnant women exposed to stressor agents exhibit increased sAA concentrations<sup>17</sup>; conversely, other study have demonstrated less marked changes in sAA levels in response to stress in pregnant *versus* non-pregnant subjects<sup>5</sup>.

Salivary cortisol and sAA have been used in medical and psychological research as physiological and psychological markers of psychosocial stress<sup>15,21-23</sup>. However, data on baseline sAA and cortisol levels during the menstrual cycle in humans are scarce, conflicting, and inconclusive in relation to changes during pregnancy<sup>5,19,20,24</sup>.

The aim of the present study was to measure serum and salivary cortisol levels, sAA and unstimulated whole saliva (UWS) flow rate in pregnant and non-pregnant women and compare these levels during each trimester of pregnancy (in the pregnant group) and during the follicular and luteal phases of the ovarian cycle (in the non-pregnant group). A secondary objective was to ascertain whether correlations exist between these variables.

## Methods

Pregnant and non-pregnant women seen at Hospital Universitário da Universidade de Brasília, in Brasília, Brazil, were invited to take part in this longitudinal study. The criteria for inclusion common to both groups were good overall health, age >18 years, no history of miscarriage during the last 2 years, no current systemic pharmacotherapy, and no smoking. Pregnant subjects were required to be in the first trimester, and non-pregnant participants were required to refrain from hormonal contraceptive use. All participants underwent an intraoral examination, interview and history-taking, and blood and UWS collection in each trimester of pregnancy (for pregnant participants) and during the follicular and luteal phases of the menstrual cycle (for non-pregnant participants). The study was conducted according to the Declaration of Helsinki and all participants provided written informed consent approval by the Universidade de Brasília School of Medicine Research Ethics Committee (#040/07).

### Case series and sample collection

In the pregnant group, first-trimester samples were collected between gestational weeks 11 and 16; second-trimester samples, between gestational weeks 18 and 22; and third-trimester samples, between weeks 32 and 36. All non-pregnant participants had regular cycles, and menstrual cycle phases were estimated on the basis of information provided during the interview. The self-reported date of onset of menses was used to calculate the follicular phase (6 to 8 days later) and luteal phase (23 to 25 days later).

Participants were instructed to arrive after at least 8 hours of fasting and to have completed their routine oral hygiene within 2 hours before sample collection. Blood samples were collected first, between 7:00 and 8:00 a.m., followed by UWS. Patients were seated on a

dental chair for 2 minutes to relax and then instructed to spit, so as to discard any detritus-containing saliva present in the oral cavity. This was defined as time point zero for collection. Participants remained seated, with eyes open and the neck and head flexed forward to facilitate “passive” flow of saliva, and were instructed to refrain from moving the tongue, cheeks, or lips. UWS was collected into a 50-mL Falcon® polypropylene tube. Overall collection time was 6 minutes. Samples exhibiting reddish discoloration (suggesting presence of blood) or cloudiness or turbidity (suggesting excessive epithelial cell shedding) were discarded to prevent excessive variation in cortisol and sAA levels. The collected samples were immediately sent for analysis. The UWS flow rate was expressed as volume of saliva/unit of time (mL/min).

### Laboratory analysis of cortisol and sAA levels

For measurement of cortisol levels, saliva samples were centrifuged and the supernatant set aside for analysis. Cortisol levels in serum and saliva (µg/dL) were determined by chemiluminescence assay (Immulin 2000®, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA), using reagents and calibration materials provided by the manufacturer. A 10 µL aliquot of serum/saliva was used and the calibration curve ranged from 1–50 µg/dL for cortisol. For measurement of sAA (U/L), saliva samples were diluted in distilled water to a concentration of 1:100 (1%) and processed in an Architect c8000® automated chemistry analyzer (Abbott Clinical Chemistry, Wiesbaden, Germany), using reagents and calibration materials provided by the manufacturer.

### Statistical methods

The sample size was calculated for a two-sided test to have 80% power to detect a clinically significant mean (standard deviation) difference of 4.4 (3.5) µg/dL in salivary cortisol between groups, based on previous study<sup>25,26</sup>. Alpha was set at 0.05. The total number of subjects to be recruited was 20. Nevertheless, it was assumed an attrition rate of 30%, which is to be expected in a long-term study once pregnant women are susceptible to intercurrent conditions. Furthermore, it was also considered that non-pregnant subjects were asked to attend study visits repeatedly without any health reason, nor monetary incentive to do so. Hence, it was enrolled 26 participants.

Data were analyzed in the Statistical Package for the Social Sciences (SPSS® 20.0 for Windows, SPSS Inc., IBM Group, Chicago, USA). All tests were two-sided and the significance level was set at  $p < 0.05$ . Initially, the Mann-Whitney U test was used to assess between-group differences (pregnant *versus* non-pregnant). Afterwards, within-group differences were assessed. Serum cortisol,

sAA and UWS flow rate were compared among pregnancy trimesters by means of analysis of variance (ANOVA), while the Kruskal-Wallis test was used for comparison of salivary cortisol levels. Within-group differences between the follicular and luteal phases of the ovarian cycle in non-pregnant subjects were assessed with the Wilcoxon test (salivary cortisol, sAA, and UWS flow rate) or a dependent t-test (serum cortisol). Spearman correlation coefficients were calculated for sAA, UWS flow rate, and serum and salivary cortisol.

## Results

A total of 13 pregnant (primigravida) and 13 non-pregnant women were enrolled. Three miscarriages and one dropout occurred in the pregnant group, and one control was lost to follow-up. Thus, 9 primigravidas with median age (interquartile range) of 28 years (25–31), and 12 non-pregnant women aged 29 (27–32) took part in the study. A total of 27 and 24 samples were collected for each variable per group, respectively. There were no significant between-group differences in salivary cortisol levels. However, significant within-group differences in median (interquartile range) levels were found among the non-pregnant subjects, with values of 1.0 (1.0–1.05) µg/dL in the follicular phase of the ovarian cycle *versus* 1.1 (1.0–1.48) µg/dL in the luteal phase ( $p < 0.001$ ) (Figure 1).

Median serum cortisol levels were significantly different in the pregnant and non-pregnant groups (23.8 [19.4–29.4] *versus* 12.3 [9.6–16.8] µg/dL, and also between follicular *versus* luteal phase (12.7 [10.2–18.7] *versus* 12.2 [9.1–15.1] µg/dL ( $p < 0.001$ ) (Figure 2).

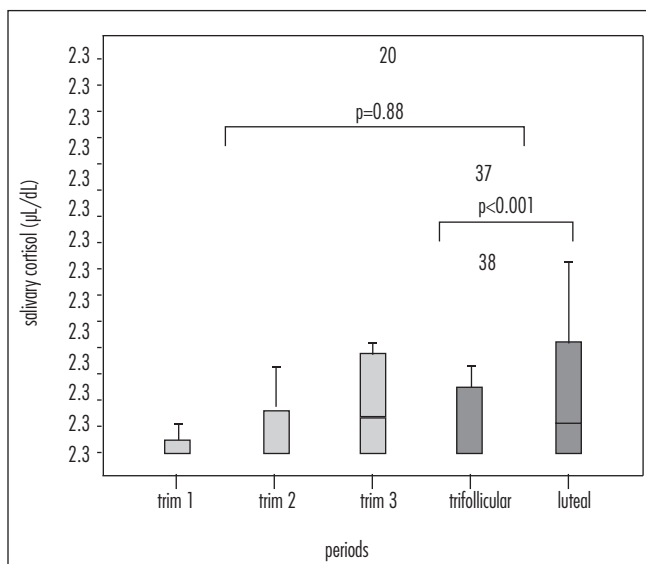


Figure 1. Between-group and within-group salivary cortisol.

There were no differences in median UWS flow rate values between pregnant and non-pregnant subjects (0.26 [0.15–0.30] *versus* 0.23 [0.20–0.32] mL/min), and no within-group differences among trimesters of pregnancy or between the follicular and luteal phases of the ovarian cycle (data not shown).

Levels of sAA were significantly different between the pregnant and non-pregnant groups (56.7 [30.9–82.2] *versus* 31.8 [18.1–53.2] U/L,  $p < 0.001$ ) and between the follicular and luteal phases in the non-pregnant group ( $p < 0.001$ ) (Figure 3).

Significant Spearman correlations were found between salivary and serum cortisol levels ( $p = 0.02$ ) and between salivary cortisol and sAA ( $p = 0.01$ ) (Table 1).

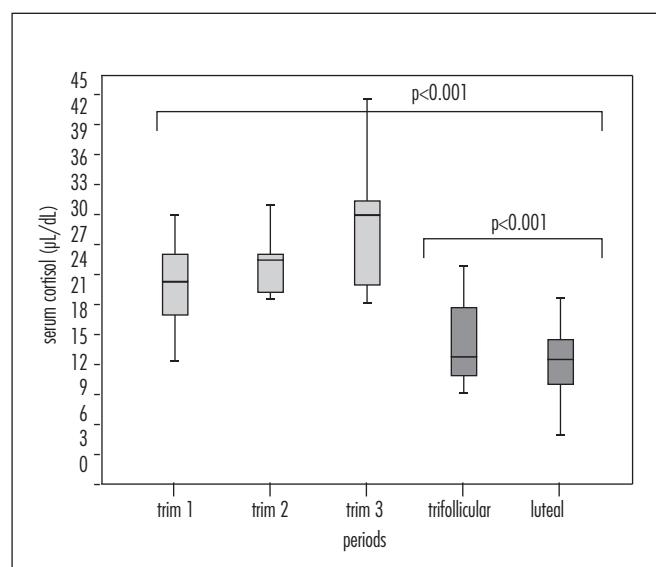


Figure 2. Between-group and within-group serum cortisol.

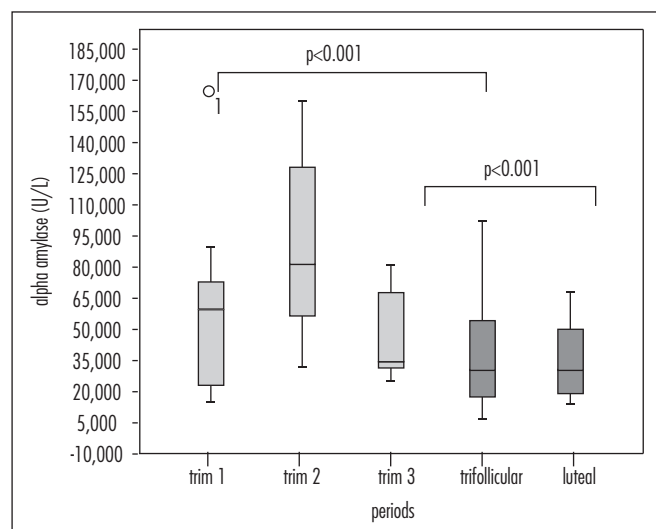


Figure 3. Between-group and within-group sAA.

Table 1. Spearman correlation coefficients

|                |                         | Salivary cortisol | Serum cortisol | Alpha-amylase | UWS flow rate |
|----------------|-------------------------|-------------------|----------------|---------------|---------------|
| Spearman's rho | Correlation coefficient | 1.000             | 0.324*         | -0.351*       | -0.080        |
|                | Sig. (2-tailed)         | —                 | 0.020          | 0.011         | 0.575         |
| Serum cortisol | Correlation coefficient |                   | 1.000          | 0.135         | -0.155        |
|                | Sig. (2-tailed)         |                   | —              | 0.343         | 0.277         |
| Alpha-amylase  | Correlation coefficient |                   |                | 1.000         | 0.010         |
|                | Sig. (2-tailed)         |                   |                | —             | 0.943         |

\*Correlation is significant at the 0.05 level (2-tailed).

UWS: unstimulated whole saliva.

## Discussion

Measurement of salivary cortisol levels has been widely used as an alternative to quantitation of this hormone in plasma or serum. Saliva samples are readily obtained and can be collected several times a day, allowing dynamic assessment of free cortisol secretion<sup>5,6</sup>. Circulating unbound cortisol is quickly transported to saliva by passive diffusion, to the extent that some studies report strong correlations between salivary cortisol levels and free (unbound) cortisol concentrations in plasma and serum<sup>6,26,27</sup>.

However, if salivary cortisol levels are to be used for diagnostic purposes in clinical practice, analytical methods must be standardized and cutoff values defined on the basis of normal population-wide control samples to serve as a reference range for testing. Research and retail laboratories should validate their salivary cortisol assays before making them available to clinicians<sup>2,28</sup>.

The interrelatedness between serum and salivary cortisol levels in non-pregnant women and men appears to be the same. However, women in the third trimester of pregnancy and those on oral contraceptives exhibit markedly increased serum cortisol levels, but near-normal salivary cortisol<sup>27</sup>.

The linear correlation between serum free cortisol and salivary cortisol is usually very strong, independent of changes in CBG concentrations, and similar across all groups: men, pregnant women, non-pregnant women, and oral contraceptive users<sup>27</sup>.

Conversely, some authors believe these parameters should be interpreted cautiously<sup>29,30</sup>, as salivary cortisol concentrations do not correlate linearly with serum levels in some cases. This nonlinearity in association between total salivary and serum cortisol may be attributable to the presence of CBG in plasma. CBG concentrations may be increased during oral contraceptive use and in certain physiological conditions, such as pregnancy<sup>27,30</sup>.



This might explain the fact that, in the present study, salivary and serum cortisol levels were significantly correlated from a statistical but not from a clinical standpoint. It bears stressing that blood and saliva sampling took place in a short period of time (30 minutes at most) so as to prevent circadian influences on measurement of the parameters of interest.

When both CBG and cortisol concentrations are increased simultaneously, as in pregnancy, salivary cortisol levels appear to remain within normal limits, although above the baseline levels found in non-pregnant women<sup>31,32</sup>. Studies have shown that salivary cortisol levels increase gradually, eventually exceeding the upper limit of normal, during the second half of pregnancy<sup>8,26,33</sup>. In the present study, there were no significant between-group differences in salivary cortisol levels. Serum cortisol levels, however, were higher in pregnant *versus* non-pregnant women. These findings are consistent with the literature<sup>9,26,31,32</sup>.

In the present study, significant differences were found between salivary cortisol levels in the follicular and luteal phases of the ovarian cycle, with higher values found in the latter. A previous study also found that salivary cortisol response patterns were more evident in the luteal phase, although the subjects were exposed to psychosocial stress, unlike the participants of the present study, who were not subjected to any external stressors<sup>23</sup>. A review of the literature did not yield any evidence that would explain this difference.

It has been suggested that salivary free cortisol levels are independent of salivary flow<sup>2</sup>. Accordingly, salivary flow rate and salivary cortisol concentration were unrelated in the present sample. This makes salivary cortisol a good biomarker, as it is unaffected by salivation itself. Although measurement of stimulated saliva is useful for assessment of the functional capacity of the salivary glands, unstimulated saliva is the predominant form during most of the day as well as during sleep, and plays an important role in the maintenance of oral health<sup>24,34</sup>. Furthermore, research suggests that if analysis of the biochemical components of saliva is to be feasible in outpatient clinical practice, whole<sup>27</sup> and unstimulated<sup>28</sup> saliva must be used; therefore, UWS was used in the present study.

Although salivary free cortisol has been proposed as a useful parameter for assessment of pituitary-adrenal function, an appropriate biomarker that reflects sympathoadrenal medullary activity has yet to be found. In this context, sAA has been suggested as a potential parameter for determination of autonomic activity and, thus, a reliable and noninvasively quantifiable indicator of stress-related changes in the human body<sup>15</sup>. The salivary glands are innervated by sympathetic and parasympathetic nerve fibers alike, so that salivary secretion occurs in response to neurotransmitter-mediated stimulation<sup>35</sup>.

As this biomarker is produced locally in the oral cavity, it is found in high concentrations in saliva as compared with other markers, such as cortisol, which is a component of blood serum, produced by the adrenal gland, and transported to the saliva via ultrafiltration in the salivary glands<sup>36</sup>.

There is no established correlation between sAA and salivary or blood levels of cortisol in the literature<sup>13,15</sup>. In the present study, salivary cortisol levels correlated with sAA. However, there was no correlation between sAA and serum cortisol. This may be attributable to production in different sites<sup>18,22</sup>. Nevertheless, the potential interrelatedness between these two parameters warrants further investigation.

Prior studies diverge as to the sAA response to pregnancy. Comparison between UWS collected from pregnant and non-pregnant women showed increased sAA levels in the first and second trimesters of pregnancy as compared to near-term and non-pregnant women<sup>24</sup>. Other research, however, failed to find any significant differences in sAA concentrations during pregnancy<sup>19</sup>. Supporting this finding, no significant changes in sAA levels during gestation were observed in the present study. However, sAA activity was increased in pregnant *versus* non-pregnant subjects. We also observed greater sAA activity in the luteal phase of the ovarian cycle as compared with the follicular phase. To the best of our knowledge, no other studies have assessed sAA levels during the distinct phases of the menstrual cycle. One previous study found high levels of sAA, but not of salivary cortisol, to be associated with a reduction in female fertility<sup>18</sup>. The mechanism whereby sAA might reduce fertility remains unknown<sup>18</sup>.

Measurement of salivary flow rate plays an important role in the interpretation of changes in salivary protein. However, a previous study showed that no association whatsoever exists between sAA activity and salivary flow rate<sup>37</sup>. In addition, authors have confirmed that sAA measurement can be performed without assessment of salivary flow, as the latter does not interfere with sAA activation<sup>22,38</sup>. The present study found no correlation between sAA and salivary flow rate and no correlations between cortisol and salivary flow rates measured in pregnant and non-pregnant women, which confirms that neither cortisol nor sAA levels are significantly altered as a function of salivary flow rate.

Nevertheless, some limitations of this study should be noted. The small sample size precludes generalization of findings to other populations. Furthermore, in the non-pregnant group, the timing of ovarian cycle phases was estimated solely on the basis of self-provided information, and not evaluated by any tests.

However, in view of the foregoing, it is essential that investigators or clinicians be aware of potential differences attributable to distinct assay methodologies, to enable proper interpretation of reference ranges. Saliva-based

diagnostics still require further research for standardization of analytical methods, validation of results, and definition of analyte reference ranges in a series of populations before they can be made available to clinical practice.

In conclusion, serum cortisol and sAA levels are higher in pregnant than in non-pregnant women. During the ovarian cycle, salivary cortisol levels increase in the luteal phase, whereas serum cortisol and sAA levels decline.

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